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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: RECOMBINANT IGF BINDING PROTEIN (IBP-I)

(1)

A-P-W-Q-C-A-P-C-S-A-E-K-L-A-L-C-P-P-V-S-A-S-C-S-E-V-T-R-S-A-G-C-G-C-C-P-M-C-A-L-P-L-G-A-A-C-G-V-A-T-A-R-C-A-R-G-L-S-C-R-A-L-P-G-E-Q-Q-P-L-H-A-L-T-R-G-Q-G-A-C-V-Q-E-S-D-A-S-A-P-H-A-A-E-A-G-S-P-E-S-P-E-S-T-E-I-T-E-E-E-L-L-D-N-F-H-L-M-A-P-S-E-E-D-H-S-I-L-W-D-A-I-S-T-Y-D-G-S-K-A-L-H-V-T-N-I-K-K-W-E-P-C-R-I-E-L-Y-R-V-V-E-S-L-A-K-A-Q-E-T-S-G-E-E-I-S-K-F-Y-L-P-N-C-N-K-N-G-F-Y-H-S-R-Q-C-E-T-S-M-D-G-E-A-G-L-C-W-C-V-Y-P-W-N-G-K-R-I-P-G-S-P-E-I-R-G-D-P-N-C-Q-M-Y-F-N-V-Q-N-

(57) Abstract

An IGF binding protein which has the amino acid sequence (I), or an equivalent modification thereof, such as a glycosylated modification. Further is indicated a DNA-sequence, coding for the protein, an expression vector and a pharmaceutical preparation containing the protein. The protein is effective as a potentiator for the function of IGF-compounds.

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## Recombinant IGF binding protein (IBP-1)

The present invention relates to an Insulin-like Growth Factor Binding Protein, IBP-1, having a molecular weight of about 28 kD, derived from human placenta/endometrium, and equivalent modifications thereof.

The invention further relates to a DNA-structure, coding for the IBP-1, expression vectors containing this DNA-structure and prokaryotic or eucaryotic cells comprising such a vector.

The invention still further relates to pharmaceutical preparations comprising IBP-1.

IGF or Insulin like Growth Factor is synonymous with somatomedins. The family of somatomedins are members of a group of polypeptides derived from the insulin gene. The gene products include insulin, insuline-like growth factor (IGF)I and II, relaxin and the B-unit of nerve growth factor (NGF) (Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b; Bradshaw 1978; Isaacs et al., 1978). IGF elicits classical insulin effects on all target tissues of insulin, i.e. IGF increases glucose metabolism of adipose tissue and stimulates lipid, glycogen, and protein synthesis. IGF also stimulates DNA synthesis in certain cell types. This feature reflects the capacity of IGFs to induce cell proliferation and promote organ growth in vivo. Furthermore, IGF acts on differentiation of mesenchymal cells (Froesch et al., 1985).

IGF-I and IGF-II are unique in that they are complexed to specific binding proteins in plasma (Smith, 1984). At least two different binding proteins have been iden-

tified in adult human serum, namely (1) binding protein 53 (BP-53) a GH dependent binding protein, believed to be derived from the 150 kD complex which carries most of the endogenous IGF peptides, (2) IBP-1, an IGF binding protein of about 30-40 kD which is tissue specifically expressed in endometrium and liver and accounts for most of the unsaturable binding sites in plasma. While the 53 kD-binding protein is under GH control the 30-40 kD species appears to be expressed 10 in a GH independent way.

The lower molecular weight binding protein was initially identified in human amniotic fluid and has been purified and characterized (Chochinov et al., 1977; Drop et al., 1979; Drop et al., 1982). This 30-40 kD IGF binding protein appears to be identical to binding proteins that have been purified from human serum and the human hepatoma cell line, HEPG2 (Drop et al., 1984a; Povoa et al., 1984; Povoa et al., 1985). Povoa et al showed that the NH<sub>2</sub>-terminal amino acid sequence of 15 the binding protein found in amniotic fluid and from the HEPG2 cell line are similar (Povoa et al., 1985).

Placental protein PP12, a protein originally isolated from human placenta, was found to bind IGF as well as to have an identical NH<sub>2</sub>-terminal amino acid sequence 20 (Koistinen et al., 1986).

As to the biological function of IGF-binding protein both stimulatory and inhibitory effects have been described.

30 Stimulatory effects of IGF-binding protein has been shown in at least two cases. Clemmens et al (1986) showed increased binding to fibroblast and smooth muscle cell surface receptors of IGF in complex with its binding protein.

Inhibitory effects of IGF-binding protein on various IGF actions in vitro, including stimulation of glucose transport by adipocytes, sulphate incorporation by chondrocytes and thymidine incorporation in fibroblasts

5 have been described (Zapf et al., 1979; Drop et al., 1979; Ooi et al., 1986). In addition, inhibitory effects of IGF-binding proteins on growth factor mediated mitogen activity in normal cells (cartilage assay, Drop, thesis, 1983).

- 10 According to the invention, the IGF-binding protein has the following amino acid sequence:

Ala-Pro-Trp-Gln-Cys-Ala-Pro-Cys-Ser-Ala-Glu-Lys-Leu-Ala-  
Leu-Cys-Pro-Pro-Val-Ser-Ala-Ser-Cys-Ser-Glu-Val-Thr-Arg-  
Ser-Ala-Gly-Cys-Gly-Cys-Cys-Pro-Met-Cys-Ala-Leu-Pro-Leu-  
Gly-Ala-Ala-Cys-Gly-Val-Ala-Thr-Ala-Arg-Cys-Ala-Arg-Gly-  
Leu-Ser-Cys-Arg-Ala-Leu-Pro-Gly-Glu-Gln-Gln-Pro-Leu-His-  
Ala-Leu-Thr-Arg-Gly-Gln-Gly-Ala-Cys-Val-Gln-Glu-Ser-Asp-  
Ala-Ser-Ala-Pro-His-Ala-Ala-Glu-Ala-Gly-Ser-Pro-Glu-Ser-  
Pro-Glu-Ser-Thr-Glu-Ile-Thr-Glu-Glu-Leu-Leu-Asp-Asn-  
20 Phe-His-Leu-Met-Ala-Pro-Ser-Glu-Glu-Asp-His-Ser-Ile-Leu-  
Trp-Asp-Ala-Ile-Ser-Thr-Tyr-Asp-Gly-Ser-Lys-Ala-Leu-His-  
Val-Thr-Asn-Ile-Lys-Lys-Trp-Lys-Glu-Pro-Cys-Arg-Ile-Glu-  
Leu-Tyr-Arg-Val-Val-Glu-Ser-Leu-Ala-Lys-Ala-Gln-Glu-Thr-  
Ser-Gly-Glu-Glu-Ile-Ser-Lys-Phe-Tyr-Leu-Pro-Asn-Cys-Asn-  
25 Lys-Asn-Gly-Phe-Tyr-His-Ser-Arg-Gln-Cys-Glu-Thr-Ser-Met-  
Asp-Gly-Glu-Ala-Gly-Leu-Cys-Trp-Cys-Val-Tyr-Phe-Trp-Asn-  
Gly-Lys-Arg-Ile-Pro-Gly-Ser-Pro-Glu-Ile-Arg-Gly-Asp-Pro-  
Asn-Cys-Gln-Met-Tyr-Phe-Asn-Val-Gln-Asn

- 30 The complete nucleotide sequence of the corresponding cDNA sequence was shown to have the structure depicted in claim 5.

The protein according to the invention may be used as an effective potentiator for the functioning of

- somatomedins. The effect can be mediated through the firm binding between the somatomedins and their binding proteins under physiological conditions. Such complexed somatomedins together with their binding proteins are protected against undue proteolysis, causing a significant increase of the biological half life of somatomedins. Furthermore, this IGF-binding protein or modifications hereof might function as a potent carrier of IGF to its local sites of action.
- 10 The studies of Clemons (1986) demonstrate the potential usefulness of this IGF-1 binding protein or modifications hereof in fertilization and in potentiation of growth of connective tissue and muscle cells in tissue repair.
- 15 As IGF-binding protein, or modifications thereof, such as alpha 1 PEG, are the major secretory soluble protein of decidual cells of the endometrium, IBP-1 may have an important function in restricting trophoblast invasion into the endometrium during placental development. Furthermore, the inhibitory function of IBP-1 in cellular proliferation assays and the unexpected direct inhibitory effect of IBP-1 on the oestrogen response on certain cancer cells make IBP-1 or modifications hereof a potential anticancer reagent with local growth inhibitory effect.
- 20
- 25 The invention is explained more in detail in the following description with reference to the drawing, in which fig. 1 illustrates a restriction map and sequence strategy for human 28 kD IGF binding protein cDNA clones,
- 30 fig. 2 shows the nucleotide and deducted amino acid sequence of human IGF binding protein where differences between the placental cDNA sequence and the liver cDNA sequence are shown in parenthesis, and

fig. 3 represents an SDS/PAGE analysis of culture media of COS-1 cells transfected with pSV19, pSV4, pSV4Inv and untransfected COS-1 cells.

The cDNA encoding IGF-BP was obtained by screening  
5 a human placental and a human hepatoma (HEPG-2) cDNA-expression library with a polyclonal antibody to human amniotic fluid binding protein (Drop et al., 1984a).

Restriction analysis indicated that the clones isolated from the placenta library and the clones isolated from  
10 the HEPG2 library were colinear (fig. 1), supporting their candidacy as IGF binding protein clones. The composite restriction map is shown at the top of the figure 1. A putative leader sequence is shown in front of the open box representing translated regions. Each  
15 arrow shows the direction and extend of sequencing by chain termination. Four different clones are aligned. p4 and p19 originate from the placental cDNA library, while w61 and w85 originate from the HEPG2 cDNA library.  
20 (E = EcoRI, P = PstI, B = BamHI, H = HindII, S = SstI, X = XbaI, N = Ncol).

The complete nucleotide sequence of the cDNA insert of one of the clones isolated (p19) was determined. The 1421 nucleotide sequence shown in fig. 2 contains a 5' untranslated region of 52 nucleotides followed  
25 by an ATG codon and an open reading frame of 776 nucleotides. The potential initiation codon is flanked by 5 sequences matching Kozak's criteria for an initiation codon (Kozak, 1986). At the 3' end the open reading frame is flanked by a translation termination codon (TGA) and a 569 nucleotides long 3' untranslated sequence.  
30

The open reading frame in cDNA clone p19 has a coding capacity for a protein of 259 residues also shown in

figure 2 (by the one-letter code), with a calculated Mw of 28,172 daltons. The initiation methione is the first amino acid of a 24-residue highly hydrophobic peptide sequence (underlined), representing the sequence of a putative signal sequence necessary for transfer of the nascent polypeptide sequence into the membranes of the endoplasmatic reticulum. A favourable signal peptidase cleavage site (ala-gly) occurs immediately N-terminally of the alanine residue at pos +1 (von Heijne, 1987). The NH<sub>2</sub>-terminus of the predicted mature protein is identical to the chemically determined NH<sub>2</sub>-terminus described for the IGF-binding protein isolated both from amniotic fluid (Povoa et al., 1984), and from the HEPG2 cell line (Povoa et al., 1985) and from serum (Baxter et al., 1987).

Omitting the signal peptide sequence, the M<sub>r</sub> of this gene product is predicted to be 2,350 daltons. The M<sub>r</sub> of serum IGF binding protein is about 28,000 daltons (Baxter et al., 1987). The difference is believed to be accounted for by glycosylation of the IGF binding protein (Bohn et al., 1980; Koistinen et al., 1986).

The amino acid sequence did not disclose N-linked glycosylation sites (N-T, N-S). However, at least five potential O-linked glycosylation sites were found in the NH<sub>2</sub>-terminal of the molecule. A RGD sequence in the COOH terminal part of the IBP-1 protein has been found. Such a short sequence is considered to be important for cellular attachment of matrix proteins, such as fibronectin, vitronectin and von Willebrand factor, to receptors of the integrin family.

Amino acid homology with other known proteins and peptides were determined by searching the NFBR data base, version 12.0 and 26.0. The IGF-BP protein did not show

any significant protein homology, indicating that IGF-BP is a unique protein. In particular, comparison of amino acid sequence of IGF-I, IGF-II, Insulin, Type I IGF receptor and the type II IGF receptor amino acid sequences revealed no homologous domains. In addition, no homology was found with the reported NH<sub>2</sub>-terminal amino acid sequence for the high molecular weight IGF binding protein (Baxter et al., 1986).

It was further possible to express IBP-1 in mammalian cells. Expression vectors, pSV19, pSV4 and pSV4Inv were constructed by inserting the full length clones p4 and p19 in the expression vector pSV328. The vectors pSV4, pSV19 and pSV4Inv, in which the cDNA insert is in 3' - 5' orientation, were transfected to COS-1 cells. By use of SDS/PAGE analysis cell culture media from COS-1 cells transfected with pSV19 (lane A), pSV4 (lane B), and amniotic fluid (lane C) were analysed. The IGF binding proteins were made visible by immuno staining as described for the screening of the cDNA libraries. In culture media of pSV4 and pSV19 transfected COS-1 cells in which the gene is in the correct orientation a protein of 32 kD being immunologically indistinguishable from the IGF binding protein from amniotic fluid (fig. 3) was detected. In all culture media a band was visible which reacted with the 35 kD SMBP antibody but which was absent in the culture medium from untransfected COS-1 cells.

Although IBP-1 successfully has been expressed in COS-1 cells the lack of N-linked glycosylation sites in the putative protein also favour expression in yeast and bacteria to increase the IBP-1 production to be used in a variety of therapeutic compositions.

The invention provides therapeutic compositions comprising IBP-1 or derivatives thereof and pharmacologically

acceptable excipients. Such compositions including the IGF-binding protein or derivatives hereof according to this invention have many therapeutic uses involving the physiological functions of somatomedins.

- 5       The IGF-binding protein of the invention may be formulated as pharmaceutical preparations comprising the IGF-binding protein of the invention together with the usual excipients. Pharmaceutical preparations according to the invention may be in the form of suspension  
10      or solutions for parenteral administration, e.g. i.v., s.c., i.m., implants, subcutaneous or interveneous administration or administration through the mucosa, e.g. oral, nasal, buccal, sublingual or rectal administration or transdermal administration.
- 15      For example in cases where somatomedins have to be transported to specific target tissues in a way where the physiological halflife of the somatomedins has to be increased by complexing IBP-1 described in this invention to IGF-1 and IGF-2. In accordance with this invention a slow release of active IGF-1 or 2 from such complexes would ascertain a constant level of somatomedins either locally or systemically dependent upon the way of administration. IBP-1 describes in this invention hereby abolishes the potent mitogenic effect of the somatomedins that administrated in high dosis, i.e. intra venously, would cause unwanted local cellular proliferations in a variety of cells like fibroblasts, muscle cells and endothelial cells.
- 25      However, the IBP-1 described by this invention administered together with IGF-1, IGF-2 and other growth factors or formulated as common preparations for topical use (such as PDGF, EGF, FGF, TGFalpha or TGFbeta) employed in therapeutical devices to be used in healing of wounds or in treatment of oeteoporosis and in healing
- 30

of bones might be valuable for a steady and controlled release of the somatomedins in such therapeutical devices.

Such preparations may optionally be administered in  
5 the form of combination preparations e.g. comprising  
IBP-1 and IGF-1, IBP-1 and IGF-2 or IBP-1, IGF-1 and  
IGF-2.

In general, IBP-1 according to this invention might  
turn out to exhibit a potent regulatory function in  
10 the release of IGF-1 and/or IGF-2 in future treatment  
of injuries or other malfunctions that requires increased  
IGF-1 and/or IGF-1 levels.

On the other hand IBP-1 or derivatives thereof according  
to this invention might be useful in therapy of the  
15 proliferation of certain cancers characterized by pro-  
ducing somatomedins in high amounts thus inhibiting  
the autocrine/paracrine physiological stimulation of  
unwanted cellular proliferation in cancers like chondro-  
sarcomas, fibrosarcomas, and mammacarcinomas.

20 Furthermore, the IBP-1 or derivatives hereof described  
in this invention is useful for the production of anti-  
bodies. Such mono- or polyclonal antibodies are suitable  
for developing immunological methods like immunohisto-  
chemical analysis of IBP-1 in tissues and for developing  
25 ELISA for IBP-1 quantitation. Such ELISA will prove  
valuable for early screening the levels of IBP-1 in  
patients with altered IGF-1 and 2 levels.

Pharmaceutical preparations of this invention for s.c.  
and i.m. administration can be prepared by mixing the  
30 following constituents: IBP-1 and derivatives thereof  
together with IGF-1, IGF-2 and other growth factors,  
an isotonic agent, a buffer, a preservative and water.

After mixing the pH value of the preparation is, if necessary, adjusted to pH = 7.3.

Examples of preservatives: phenol and m-cresol. Examples of an isotonic agent: sodium chloride and glycerol.

5 Example of buffer is sodium phosphate.

Pharmaceutical preparations of this invention for trans-mucosal administration can be prepared by mixing the following constituents: IBP-1 and derivatives thereof, together with IGF-1, IGF-2 and other growth factors, 10 a buffer, an isotonic agent, a preservative, an absorption promotor and a vehicle e.g. water, cellulose, water-soluble cellulose alkylethers, crystalline cellulose, water-soluble polyacrylates or mixtures thereof.

15 Pharmaceutical preparations of this invention for trans-dermal administration can be prepared by mixing the following constituents: IBP-1 and derivatives thereof together with IGF-1, IGF-2 and other growth factors, an isotonic agent, a preservative and a vehicle e.g. a hydrophilic gel of water-soluble cellulose alkyl-ethers.

20 This invention is further explained in the following working example describing the isolation and characterization of IBP-1.

#### EXAMPLE

25 Screening the Lambda gt11 Expression Library

A human placenta cDNA library in lambda gt11 and a cDNA library of the human hepatoma cell line HEPG2 were screened with a polyclonal antibody to human amniotic fluid binding protein according to the procedure 30 described by Young and Davis (Young and Davis, 1982).

Rabbit antibody to 35 kD somatomedin binding protein SMBP isolated from human amniotic fluid was produced and purified as described by Drop et al., 1984a. The antibody was absorbed against E.coli Y1090 and lambda gt11 proteins by incubating with nitrocellulose filters that had been lifted from confluent lysis plates of E.coli Y1090/lambda gt11 induced with 10 mM isopropyl beta-D-thiogalacopyranoside (IPTG). The antibody was further absorbed against human serum albumin immobilized on nitrocellulose filters. Approximately  $4 \times 10^5$  clones of the placental library were screened and about  $0.5 \times 10^5$  of the HPEG2 library.  $3-5 \times 10^4$  plaque forming units per 150 mm Petri dish were plated on a lawn of Y1090 bacteria and incubated. After 2 hr incubation the plates were covered with nitrocellulose filters (Millipore HATF) that had been saturated with 10 mM IPTG. The plates were incubated at 37°C for 2-2.5 hrs. The filters were removed, washed with Tris-buffered saline (TBS; 10 mM Tris/HCl, pH 7.5/150 mM NaCl) at room temperature and incubated with 3% BSA in TBS for 30 min. at room temperature. Partly purified rabbit polyclonal 35 kD SMBP antibody diluted 1:125 was added to 3% BSA in TBS plus 0.02% azide, and the filters were incubated overnight at 4°C. The filters were washed and incubated for 60 min. at room temperature with horse-radish peroxidase conjugated goat anti-rabbit IgG (Tago) diluted 1:200 in 3% BSA in TBS. The filters were washed and stained with amidophenyl and naphthol AS-MX phosphate in 0.2 M Tris/HCl, pH 9.2, 10 mM MgCl<sub>2</sub> at room temperature.

Positive phages were isolated and DNA was isolated by standard methods (Maniatis et al., 1982). About 33 plaques strongly cross-reacting with the polyclonal antibody were identified in the placenta and HEPG2 cDNA library. Following re-screening inserts varying in size between 0.9-1.5 Kb were isolated and subcloned

in the vector PTZ19 from Pharmacia. All isolated clones showed cross-hybridization in a Southern blot except one clone from the placenta library and the 5 weakly hybridizing clones from the HEPG2 library.

- 5 cDNA was digested with various restriction endonucleases (BRL, NEN, Boehringer) according to the suppliers directions, electrophoresed in 0.8% agarose, and transferred to nitrocellulose filters according to the method of southern (Southern, 1975). mRNA was denatured with dimethylsulfoxide (DMSO) and glyoxal, subjected to electrophoresis in 1% agarose and transferred to nitrocellulose filters (Millipore HFTF).
- 10

Restriction fragments were subcloned in the vectors PTZ18 or PTZ19 (Pharmacia) and sequenced according  
15 to the chain termination method (Sanger et al., 1977). In regions which lacked convenient restriction sites, appropriate clones were generated by Bal 31 nuclease digestion.

#### Transfection of COS-1 Cells

- 20 The full length cDNA clones p4 and p19 were subcloned in the EcoR1 site of pSV328, which expressed cloned inserts using the simian virus 40 (SV40) early promotor (Van Heuvel et al., 1986). A DEAE-dextran procedure (McCuthchan & Pagano, 1986) followed by treatment with  
25 100  $\mu$ M chloroquine in Dulbecco's MEM (DMEM) for 4 hrs was used to transfect COS-1 cells (Gluzman, 1981). After this treatment the cells were grown 24 hrs with DMEM plus 5% foetal calf serum. Medium was removed after 72 hrs, and the cells were washed extensively with DMEM and incubated for 72 hrs with DMEM without serum. Production of 32 kD binding protein in culture media was determined using 35 kD SMBP antibody.
- 30

Purification of IBP-1

Proteins from amniotic fluid or from conditioned media were precipitated with ammonium sulphate at a final concentration of 35%. Following centrifugation the supernatant was brought to 50% ammonium sulphate. The pellet was dissolved in 45% ammonium sulphate and the final pellet was dissolved in 50 mM Tris HCl, pH 7.5 for further purification and characterization. The dissolved ammonium sulphate precipitate was further purified by reverse phase chromatography on C<sub>18</sub>. Following washings with 50 mM Tris-HCl, pH 7.5 and Tris HCl, pH 7.5 in 50% methanol the pure IBP-1 was eluted from the column with Tris-HCl, pH 7.5 in 65% methanol. The IBP-1 was precipitated overnight in a 7% Trichloroacetic-acid solution. The precipitate was dissolved in 20 mM Tris-HCl, pH 7.5, lyophilized and storred at + 20°C.

Characterization of purified IBP-1

IBP-1 binds both IGF-1 and IGF-2 with approximately the same specificity when measured in binding assays with [<sup>125</sup>I]IGF-1 and [<sup>125</sup>I]IGF-2 with or without excess of cold iGF-1 or IGF-2. The specificity of IBP-1 reaction was tested in similar assays employing such competition assays and in assays in which IBP-1 - IGF binding was visualized through the specific reaction of antibody against IBP-1. In such assays IBP-1 did only react with IGF-1 and IGF-2 but not with closely related compounds like insulin, proinsulin or truncated forms thereof.

The biological effects of purified samples if IBP-1 were tested in in vitro mitogenicity tests employing <sup>3</sup>H-thymidin incorporation into a MCF-7 mamma carcinoma cell line. The stimulatory effect of both IGF-1 and

IGF-2 on cell proliferation was inhibited by IBP-1 in a dose dependent way. The effect of IBP-1 on IGF-2 dependent cell proliferation was more pronounced than that of the effect of IBF-1.

- 5 Furthermore, in an assay employing  $^{35}\text{S}$ -Methionine incorporation into cartilage the stimulatory effect of IGF-1 and IGF-2 was abolished by IBP in the low ng range.

Examples of pharmaceutical preparations:

10 EXAMPLE 1

- 25 ng IBP-1  
10 ng IGF-1  
0.7% NaCl  
1/75M sodiumphosphate  
15 water ad 1 ml

The calculated amounts of IBP-1 and IGF-1 were dissolved and diluted in phosphate buffer containing NaCl. The pH was adjusted to 7.3-7.4.

EXAMPLE 2

- 20 25 ng IBP-1  
10 ng IGF-2  
1.6% glycerin  
1/75M sodiumphosphate  
0.01% benzalconiumchlorid  
25 0.05% sodiumedetate  
water ad 1 ml

The calculated amounts of IBP-1 and IGF-2 were dissolved and diluted in phosphate buffer containing glycerin, benzalconiumchlorid and sodiumedetate. The pH

was adjusted to 7.4

EXAMPLE 3

25 ng IBP-1

10 ng IGF-1

5 10 ng IGF-2

5% hydroxyethylcellulose

0.9% benzylalcohol

1/75M phosphate buffer

water ad 1 ml

- 10 The gel is prepared by mixing hydroxyethylcellulose  
with the waterphase containing IBP-1, IGF-1 and IGF-2.

## P a t e n t C l a i m s :

1. An IGF binding protein, comprising the following amino acid sequence:

A-P-W-Q-C-A-P-C-S-A-E-K-L-A-L-C-P-P-V-S-A-S-C-S-E-V-T-R-S-A-  
5 G-C-G-C-C-P-M-C-A-L-P-L-G-A-A-C-G-V-A-T-A-R-C-A-R-G-L-S-C-R-  
A-L-P-G-E-Q-Q-P-L-H-A-L-T-R-G-Q-G-A-C-V-Q-E-S-D-A-S-A-P-H-A-  
A-E-A-G-S-P-E-S-P-E-S-T-E-I-T-E-E-L-L-D-N-F-H-L-M-A-P-S-E-  
E-D-H-S-I-L-W-D-A-I-S-T-Y-D-G-S-K-A-L-H-V-T-N-I-K-K-W-E-P-C-  
R-I-E-L-Y-R-V-V-E-S-L-A-K-A-Q-E-T-S-G-E-E-I-S-K-F-Y-L-P-N-C-  
10 N-K-N-G-F-Y-H-S-R-Q-C-E-T-S-M-D-G-E-A-G-L-C-W-C-V-Y-P-W-N-G-  
K-R-I-P-G-S-P-E-I-R-G-D-P-N-C-Q-M-Y-F-N-V-Q-N-

or an equivalent modification thereof.

2. A glycosylated modification of the protein according to claim 1.

15 3. An IGF binding protein according to claim 2, in which one or more hydroxy groups are glycosylated.

4. A DNA sequence, coding for the IGF binding protein as defined in claim 1.

20 5. A cDNA sequence according to claim 4 in which the coding strand includes the following structure:

1 GGGCGGGCAC AGCCAGAGAG CATCGGCCGC TGTCTGCTGC TCGCGCCTGG  
 51 AGATGTCAGA GGTCGGCGTT GCTCGCGTCT GGCTGGTACT GCTCCCTGCTG  
 101 ACTGTCCAGG TCGGCGTGAC AGCCGGCGCT CCGTGGCAGT GC~~GCGCC~~CTG  
 151 CTCCGCCGAG AAGCTCGCGC TCTGCCCGCC GG~~TGTCC~~GC TCGTGCTCGG  
 201 AGGTCA~~CCCCG~~ GTCCGCCGGC TGCGGCTGTT GCCCGATGTG CGCCCTGCCT  
 251 CTGGGCGCCG CGTGC~~GGCGT~~ GGCGACTGCA CGCTGC~~GC~~CCC GGGGACTCAG  
 301 TTGCCGCCGCT CGCCGGGG AGCAGCAACC TCTGCACGCC CTCACCGECG  
 351 GCCAAGGC~~GC~~ CTGCGTGCAG GAGTCTGACG CCTCCGCTCC CCATGCTGCA  
 401 GAGGCA~~GGGA~~ GCCCTGAAAG CCCAGAGAGC AC~~GG~~AGATAA CT~~GG~~AGGAGGA  
 451 GCTCCTGGAT AATTTCATC TGATESCCCC TTCTGAAGAG GATCATTCCA  
 501 TCC~~TTT~~GGGA CGCCATCAGT ACCTATGATG GCTCGAAGGC TCTCCATGTC  
 551 ACCAACATCA AAAAAT~~GGAA~~ GGAGCCCTGC CGAATAGAAC TCTACAGAGT  
 601 CGTAGAGAGT TT~~A~~CCCAAGG CACAGGAGAC ATCAGGAGAA GAAATTCCA  
 651 AATT~~TT~~ACCT GCCAAACTGC AACAAAGAATG GATTTTATCA CAGCAGACAG  
 701 TGTGAGACAT CCATGGATEG AGAGGCGGGAG CTCTGCTGGT GCGTCTACCC  
 751 TTGGAA~~ATGGG~~ AAGAGGATCC CTGGGTCTCC AGAGATCAGG GGAGACCCCCA  
 801 ACTGCCAGAT GTATTTAAT GTACAAAACT GAAACCAGAT GAAATAATET  
 851 TCTGTCACGT GAAATATTAA AGTATATAGT ATATTTATAC TCTAGAACAT  
 901 GCACATTTAT ATATATGTAT ATGTATATAT ATATA~~GT~~AAC TACTTCTTAT  
 951 ACTCCATACA TAACTTGATA TAGAAAGCTG TTTATTTATT CACTGTAAGT  
 1001 TTATTTTTTC TACACAGTAA AAACTTGAC TATGTTAATA ACTTGTCCTA  
 1051 TGTCAATTG TATATCATGA AACACTTCTC ATCATATTGT ATGTAAGTAA  
 1101 TTGCCATTCT GCTCTTCCAA AGCTCCTGCG TCTGTTTTA AAGAGCATEG  
 1151 AAAAAT~~ACTG~~ CCTAGAAAAT GCAAAATGAA ATAAGAGAGA GTAGTTTTTC  
 1201 AGCTAGTTG AAGGAGGACG GTTAACTTG ATATTCCACC ATTCAACATT  
 1251 GATGTACATG TGTAGGGAAA GTTAAAAGTG TTGATTACAT AATCAAAAGCT  
 1301 ACCTGTEGTG ATAGTTGCCA CCTGTTAAAA TGTACACTGG ATATGTTGTT  
 1351 AACACGTGT CTATA~~ATGAA~~ AACATTTACA ATAAATTC TGCATCAA  
 1401 AAAA~~AAAAAA~~ AAAA~~AAAAAA~~ AAAA

6. An expression vector, containing the DNA sequence defined in claim 4 or 5.
7. The expression vector pSV19.
8. The expression vector pSV4.
- 5 9. A cell line or a microorganism, comprising an expression vector in accordance with claims 6, 7 or 8.
10. A pharmaceutical preparation, comprising the binding protein defined in any of the claims 1-3.

LITERATURE:

1. Baxter R.C., Martin J.L., Tyler M.I., Howden, M.E.H. Growth hormone-dependent insulin-like growth factor (IGF) binding protein from human plasma differs from other human IGF binding proteins.  
a. Biochem. Biophys. Res. Communun. 1986: 139: 1256-1261.
2. Bohn H., Kraus W. Isolierung und Charakterisierung eines neuen plazentaspezifischen Proteins (PP12). Arch. Gynecol. 1980: 229-279
3. Bradshaw R.A. Nerve growth factor. Annu. Rev. Biochem. 1978: 47: 191-216.
4. Clemons D.R., Elgin R.G., Han V.K.M., Casella S.J., D'Ercole A.J., Van Wyk J.J. Cultured fibroblast monolayers secrete a protein that alters the cellular binding of somatomedin-C/insulin-like growth factor I. J. Clin. Invest. 1986: 77: 1548-1556.
5. De Vroede M.A., Tseng, L.Y.H., Katsoyannis P.G., Nissiev S.P., Rechler M.M. Modulation of insulin-like growth factor I binding to human fibroblast monolayer cultures by insulin-like growth factor carrier proteins released to the incubation media. J. Clin. Invest. 1986: 77: 602-613.
6. Drop S.L.S., Valiquette G., Guyda H.J., Corvol M.T., Posner B.I. Partial purification and characterization of a binding protein for insulin-like activity (ILAs) in human amniotic fluid: a possible inhibitor of insulin-like activity. Acta. Endocrinol. (Copenh.) 1979) 90: 505-5118.

7. Drop S., Kortleve D., Guyda H. Isolation of a somatomedin binding protein in human amniotic fluid: development of a radioimmunoassay. *Pediatr. Res.* 1982; 16: 890894.
8. Drop S.L.S., Kortleve D.J., Guyda H.J. Isolation of a somatomedin-binding protein from preterm amniotic fluid. Development of a radioimmunoassay. *J. Clin. Endocrinol. Metab.* 1984a: 59: 899-907.
9. Elgin R.G., Busby Jr. W.H., Clemons D.R. An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-I. *Proc. Natl. Acad. Sci. USA* 1987: 84: 3254-3258.
10. Froesch E.R. Schmid C., Schwander J., Zapf J. Actions of insulin-like growth factor. *Ann. Rev. Physiol.* 1985: 47: 443-467.
11. Heijne G. von. A new method for predicting signal sequence cleavage sites. *Nucleic. Acid. Research* 1986: 14: 714-722.
12. Isaacs N., James R., Niall H. et al. Relaxin and its structural relationship to insulin. *Nature* 1978: 271: 278-281.
13. Koistinen R., Kalkkinen N., Huhtala M.L., Seppala M., Bohn H., Rutanen E.M. Placental protein 12 is a decidua protein that binds somatomedin and has an identical N-terminal amino acid sequence with somatomedin-binding protein from human amniotic fluid. *Endocrinology* 1986: 118: 1375-1378.
14. Kozak M. Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. *Proc. Natl. Acad. Sci. USA* 1986: 83: 2850-2854.

15. Maniatis T., Fritsch E.F., Sambrook J. ed. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory 1982.
16. Povoa G., Enberg G., Jörnvall H., Hall K. Isolation and characterization of a somatomedin-binding protein from mid-term human amniotic fluid.  
a. Eur. J. Biochem. 1984: 144: 199-204.
17. Povoa G., Isaksson M., Jörnvall H., Hall K. The somatomedin-binding protein isolated from a human hepatoma cell line is identical to the human amniotic fluid somatomedin-binding protein. Biochem. Biophys. Res. Commun. 1985: 128: 1071-1078.
18. Rinderknecht E., Humbel R.E. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J. Biol. Chem. 1978a: 253: 2769-2776.
19. Rinderknecht E., Humbel R.E. Primary structure of human insulin-like growth factor II. FEBS Lett. 1978b: 89: 283-286.
20. Sanger F., Nicklan A.R., Coulson J. DNA sequencing with chain-terminating inhibitor. Proc. Natl. Acad. Sci. 1977: 74: 5463.
21. Smith G.L. Somatomedin carrier proteins. Mol. Cell. Endocrinol. 1984: 34: 85-89.
22. Southern E. Detection of specific sequences among DNA fragments separated by gelelectrophoresis. J. Mol. Biol. 1975: 98: 503.

23. Young R.A., Davis R.W. Efficient isolation of genes by using antibody probes. Proc. Natl. Acad. Sci. USA 1983: 80: 1194-1198.
24. Gluzman Y., SV40-transformed simian cells support the replication of early SV40 mutants, Cell., 23 (1) 175-82, 1981, Jan/IMD-8108.
25. McCutchan J.H., Pagano J.S., Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran, J. Natl. Cancer Inst., 41 (2) 351-7, Aug. 68/IMD-6810.
26. Van Heuvel M., Bosveld I.J., Mooren A.A., Trapman J., Zwarthoff E.C., Properties of natural and hybrid murine alpha interferons, J. Gen. Virol., 67 (Pt 10) 2215-22, 1986 Oct/IMD-8701.
27. Zapf J., Schoenle E., Jagers G., Sand I., Grünwald J., Froesch E.R., Inhibition of the action of non-suppressible insulin-like activity on isolated fat cells by binding to its carrier protein. J. Clin. Invest. 1979: 63, 1077-1084.
28. Baxter R.C., Martin J.L., Binding proteins for insulin-like growth factors in adult rat serum. Comparison with other human and rat binding proteins. Biochem. Biophys. Res. Commun. 1987: 147, 408.
29. Ooi G.T., Herington A.C., Covalent cross-linking of insulin-like growth factor I to a specific inhibitor from human serum, Biochem. Biophys. Res. Commun. 1986: 137, 411.
30. Chochinov R.H., Mariz I.K., Hajek A.S., Daughaday W.H., Characterization of a protein in mid-term

human amniotic fluid which reacts in somatomedin-C  
radioreceptor assay, J. Clin. Endocrinol. Metab.  
1977;44, 902.

1/3

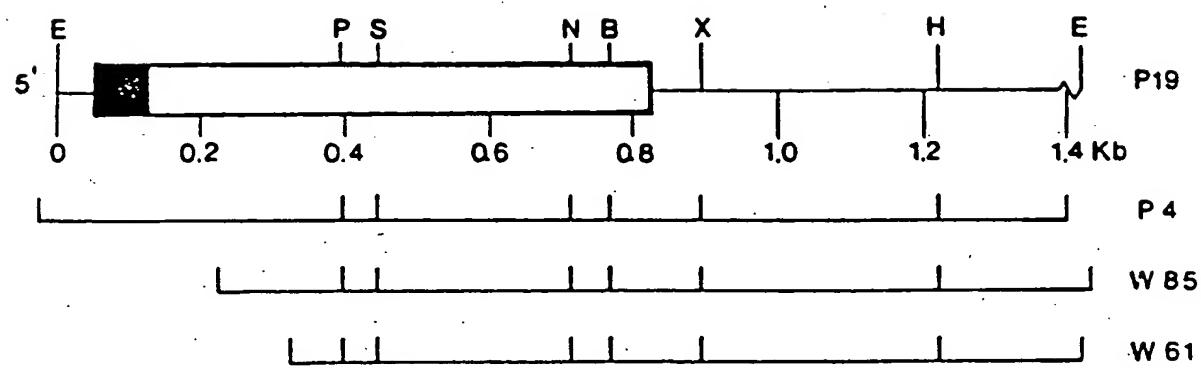


FIG.1

2 / 3

-25 M S E V P Y A P Y W I Y L -13

GGGGGGGGCACAGCCAGAGAGCATGGCCCCCTGTCGCTCCTCCCCCTGGACATTCAGAGCTCCCCCTGCCTCGCGCTGCCCTGGTACT  
10 20 30 40 50 60 70 80 90

L L T V Q V G Y T A C A P W Q C A P C S A E K L A L C P P +<sup>1</sup>  
CTCCCTCCCTGACTCTCCAGCTCGGGTGACACCCCCGGCTCCCTGGACTGCGCCCTGCTCCCCAGAAGCTCCCCCTGCCCTGGCCCGCC  
100 110 120 130 140 150 160 170 180

V S A S C S E V T R S A G C C C P M C A L P L G A A C G V 48  
GGTGTCCCCCTCGCTCGGAGGTCAACCGGCTCCCCGGCTGGGCTGTTGGCCGATGCGCCCTGCCCTGCCCTGGGGCCCGTGGGGCT  
190 200 210 220 230 240 250 260 270

A T A R C A R G L S C R A L P G E Q O P L H A L T R G Q G A 78  
GGCCACTCCACGCTGGCCCCGGGACTCACTTGGGGGGCTGCCGGGAGCAGAACCTCTGCACGCCCTCACCCGGGCAAGGGC  
280 290 300 310 320 330 340 350 360

C V Q E S D A S A P H A A E A G S P E S P E S T E I T E E E 108  
CTGGCTCCAGGAGTCTGACGGCTCCGCTCCCCATGCTGCAGAGGGACGGACCCCTGAACCCAGAGACGGAGATAACTGAGGAGGA  
370 380 390 400 410 420 430 440 450

L L D N F H L M A P S E E D H S I L W D A I S T Y D G S K A 138  
GCTCTGATAATTTCATCTGATGGCCCCTCTGAACAGCATTCATCCATCTGGCACCCATCACCTATGATGGCTCGAACGC  
460 470 480 490 500 510 520 530 540

L H V T N I K K W K E P C R I E L Y R V V E S L A K A Q E T 168  
TCTCCATCTCACCAACATCAAAATGGAGGCCCTCCGAATACAACCTACAGACTCGTAGAGAGTTAGCCAAGGCACAGGAGAC  
550 (C) 570 580 590 600 610 620 630

S G E E I S K F Y L P N C N K N G F Y H S R O C E T S M D G 198  
ATCAGGACAAGAAATTCCAAATTTCACCTGCCAACTCAACAGAATGGATTTCACCAAGACACTGTGAGACATCCATGGATGG  
640 650 660 670 680 690 700 710 720

E A G L C W C V Y P W N G K R I P G S P E I R G D P H C Q M 228  
AGAGGGGGACTCTGGCTGCCCTIACCCCTGGATGGAAAGAGGATCCCTGGCTCCAGAGATCAGGGAGACCCCCAACTCCACAT  
730 740 750 760 770 780 790 800 810

T F N V Q N 234  
GTATTTAACTCACAAACTGAAACCACATCAATAATGTTCTGTCACCTGAAATTTTAACTATATAGTATTTTAACTCTAACAT  
820 830 840 850 860 870 880 890 900

CCACCTTATATATATGTATATATATAGTAACCTACTTCTTAACTCCATACATAACTTGATATAGAAACCTGTTTATT  
910 920 930 940 950 960 970 980 990

CACTGTAACCTTCTACACACTAAACTTGACTATGTTAACTTCTTAACTCCATGAAACACTTCTC  
1000 1010 1020 1030 1040 1050 1060 1070 (T)1080

ATCATATTGTAACTAATTGCAATTGCTCTCCAAAGCTCCCTGGCTCTTTAAAGACCATGCAAAATACTGCCATGAAAAT  
1090 1100 1110 (G) 1130 1140 (C) 1150 1160 1170

GCAAAATGAAATAAGAGAGACTACTTTCACTGACTTTGAAAGGAGCACGGTTAACCTGTTAACTTCCACATTGATGACATG  
1180 1190 1200 1210 1220 1230 1240 1250 1260

TCTAGGGAAACTAAAGCTGATTACATAATCAAACTACCTGTCGATAGTTCCACCTGTTAAATCTACACTGGATATGTTCTT  
1270 1280 1290 1300 1310 1320 1330 1340 1350

AAACACGTCTCTATAATGCAACCTTACAAATAATTCTGCATGCAAAAAA  
1360 1370 1380 1390 1400 1410 1420

FIG. 2

3/3

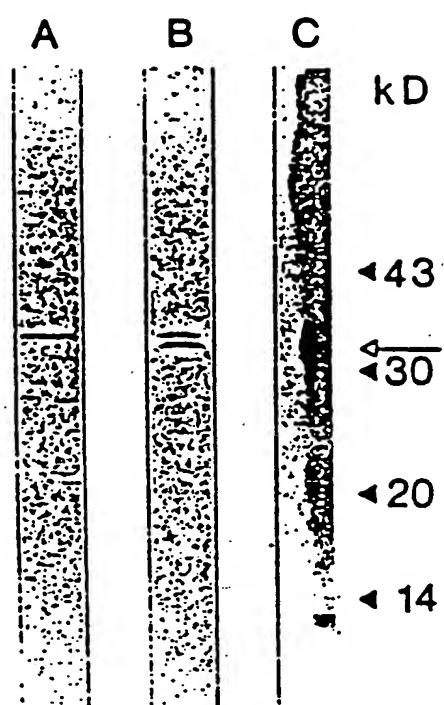


FIG. 3

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/NL 89/00008

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC<sup>4</sup>: C 07 K 13/00, C 12 N 15/00, C 12 P 21/02, A 61 K 37/02,  
IPC: //A 61 K 35/50

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>1</sup>

Classification Symbols

Classification System:

IPC<sup>4</sup>

C 07 K; C 12 N; C 12 P; A 61 K

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>2</sup>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>3</sup>

Category <sup>4</sup>	Citation of Document, <sup>5</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	EP, A, 0141326 (BEHRINGERWERKE) 15 May 1985, see the whole document --	1-3,10
X	Biochemical and Biophysical Research Communications, vol. 141, no. 1, 23 November 1986, Academic Press, Inc. (US) M.L. Huhtala et al.: "Biologically active domain in somatomedin-binding protein", pages 263-270 see the whole article --	1-3,10
P,X	Molecular Endocrinology, vol. 2, no. 5, May 1988 The Endocrine Society Y.L. Lee et al.: "Insulin-like growth factor (IGF) binding protein complementary deoxyribonucleic acid from human HEP G2 hepatoma cells: predicted protein sequence suggests an IGF binding domain different from those of the IGF-I and IGF-II receptors" pages 404-411 see the whole article --	1-10

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  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier document but published on or after the international filing date
  - "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

20th June 1989

Date of Mailing of this International Search Report

11.07.89

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

P.C.G. VANDERPUTTIEN

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document with indication where appropriate of the relevant passages	Relevant to Claim No
	<p>P,X Biochemical and Biophysical Research,  vol. 152, no. 3, 16 May 1988  Academic Press, Inc.  M.T. Brewer et al.: "Cloning  characterization, and expression of a  human insulin-like growth factor  binding protein",  pages 1289-1297  see the whole article</p> <p>--</p>	1-10
	<p>P,X Embo Journal, vol. 7, no. 8, 1988  IRL Press Ltd. Oxford (GB)  A. Brinkman et al.: "Isolation and  characterization of a cDNA encoding  the low molecular weight insulin-like  growth factor binding protein (IBP-1)  pages 2417-2423  see the whole article</p> <p>--</p>	1-10
	<p>P,X FEBS-Lett., vol. 236, no. 2, August 1988,  Elsevier Science Publishers B.V.  Biomedical Division  M. Julkunen et al.: "Primary structure  of human insulin-like growth factor-  binding protein/placental protein 12  and tissue-specific expression of its  mRNA", pages 295-302  see the whole article</p> <p>--</p>	1-10
	<p>P,X Nucleic Acids Research, vol. 16, no. 17,  1988, IRL Press Ltd. Oxford (GB)  U. Grundmann et al.: "Cloning of cDNA  encoding human placental protein 12  (PP12):binding protein for  IGF I and somatomedin"  page 8711  see the whole article</p> <p>-----</p>	1-10

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.

NL 8900008

SA 27516

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EPO file on 05/07/89.  
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0141326	15-05-85	DE-A-	3338480	02-05-85
		AU-B-	569566	04-02-88
		AU-A-	3452784	02-05-85
		CA-A-	1234100	15-03-88
		DE-A-	3473279	15-09-88
		JP-A-	60104015	08-06-85
		US-A-	4592863	03-06-86